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Role of sensitisation to testosterone in the early development of aggression in the black-headed gull

Ros, Albert Frank Huáscar

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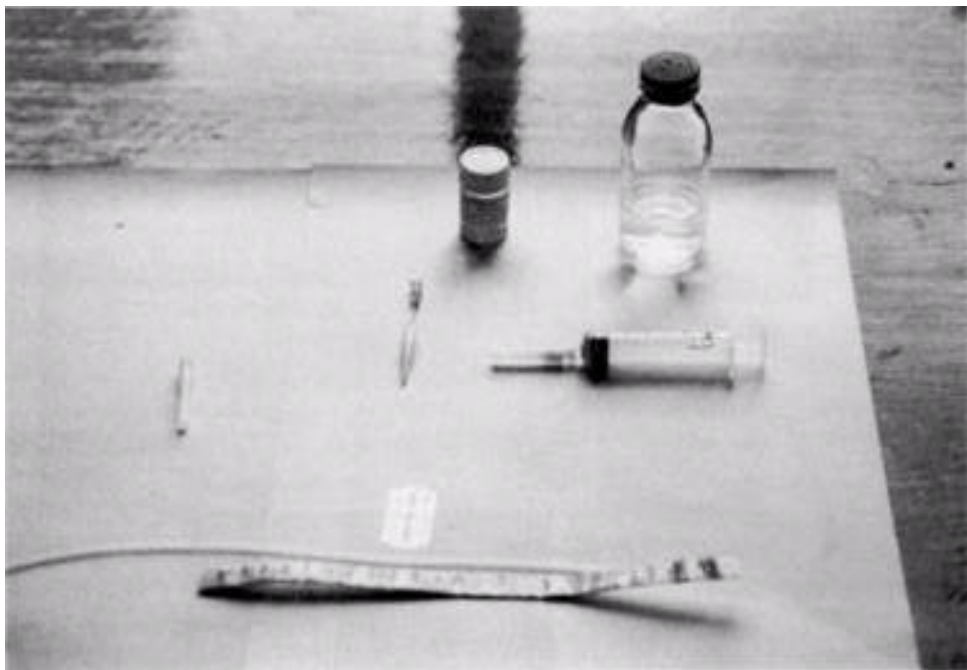
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Part 1

Testosterone and aggressive behaviour





Things that you need to make a testosterone tube...

Chapter II

Social stimuli, testosterone, and aggression in gull chicks: support for the challenge hypothesis

Albert F.H. Ros, Steph J. Dieleman* and Ton G.G. Groothuis

*Dept. of Herd Health and Reproduction University of Utrecht,
Yalelaan 7, 3584 CL Utrecht, The Netherlands

Abstract

We tested the challenge hypothesis for chicks of the black-headed gull, by analysing the influence of the interaction between aggressive challenges and testosterone on aggression in black-headed gull chicks. In the first experiment small families of three to five chicks were either kept isolated or housed together with other families (grouped condition). Basal levels of testosterone were elevated in chicks of the grouped condition only and these chicks were also more responsive with aggressive behaviour to a standard aggressive challenge (confrontation with a stuffed adult) than the chicks in the isolated condition. After the initial stage of territory establishment in the grouped condition, levels of testosterone decreased. Despite this, chicks in the grouped condition remained relatively highly responsive with aggressive behaviour to the challenge. In a second experiment this change in the relation between aggression and levels of testosterone was investigated. Young chicks in isolated families were treated for 10 days with testosterone or sham treated. Testosterone-treated birds showed higher levels of aggressive behaviour even after termination of treatment, than sham-treated birds. After termination of treatment testosterone showed low basal levels but was elevated shortly after challenge regardless of pretreatment. Therefore, we conclude that testosterone increases the sensitivity to the short lasting elevation of the hormone that occurs during the challenge. In this way exposure to testosterone (that may be detrimental for development) is minimised while birds remain able to defend territories. Our results indicate that the challenge hypothesis as established for adult birds, is also applicable for aggressive behaviour in young birds outside the sexual context. Furthermore it suggests that a phase of priming with testosterone is necessary to obtain high behavioural responsiveness to a challenge.

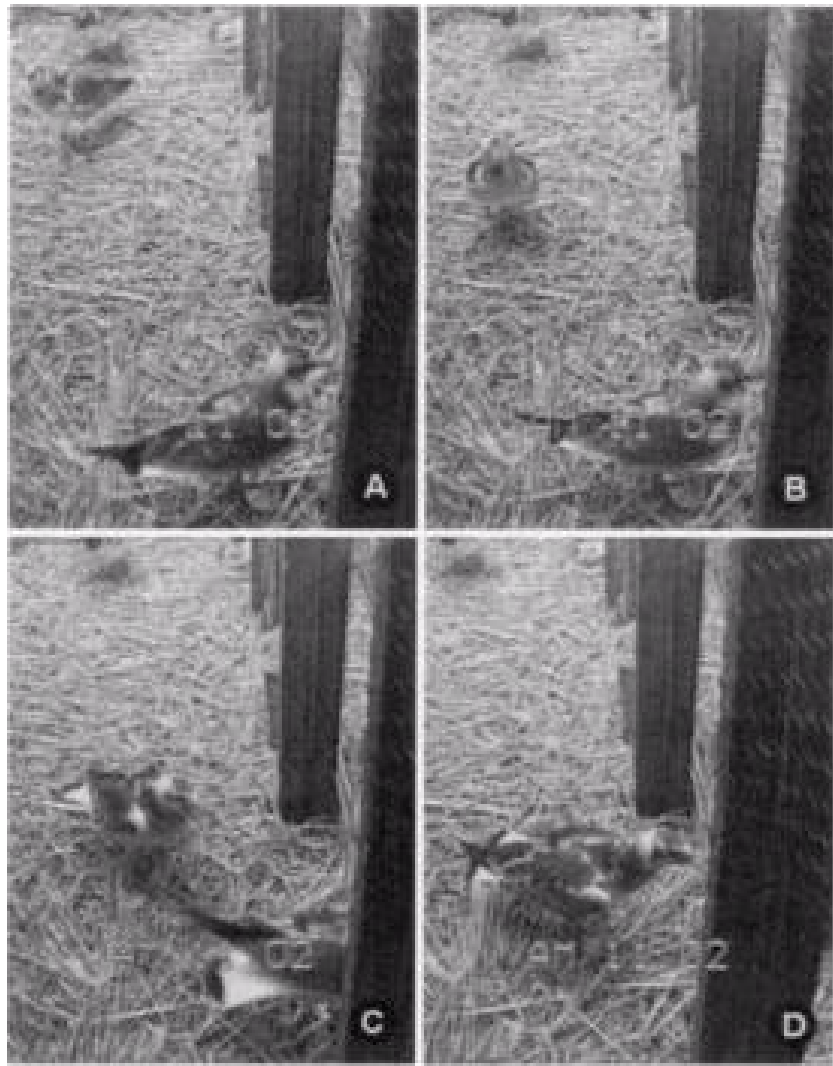
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Introduction

Testosterone is considered to be the most important hormone preparing the animal for a high level of social competition. In birds, testosterone facilitates aggressive behaviour and increases muscle growth, whereas it inhibits parental care, fat deposition, and moult (reviewed in Ketterson *et al.* 1996). Clearly some of these effects are detrimental to the animal in the long run (Dufty 1989, Moss *et al.* 1994, Ketterson *et al.* 1996). As a consequence, testosterone production should be tuned to the period in which the animal faces important social challenges. Indeed the pattern of testosterone secretion in the course of the season seems to be related to the type of social system of a species (Wingfield *et al.* 1990). In polygamous species, males are generally not involved in parental care, face high competition throughout the breeding season, and have high basal levels of testosterone. In monogamous species, high basal levels of testosterone are present only during a restricted period when territorial establishment and mate selection take place. Basal levels of testosterone are low when parental care is provided although males still respond aggressively to intruders at the territory. Several studies demonstrated that this aggressive responsiveness to a challenge is facilitated by a rapid temporary release of testosterone (Harding 1979, Ramenofsky 1984, Wingfield 1985, Hegner and Wingfield 1987) and this was generalised as a common mechanism for aggressive behaviour during the sexual period in the challenge hypothesis (Wingfield *et al.* 1990).

So far the evidence in support of the challenge hypothesis comes exclusively from studies on adult birds that provide parental care. However, in several bird species young birds also perform aggressive behaviour and do this in a non sexual context (Cash and Evans 1986, Mock *et al.* 1987, Drummond and Osorno 1992). Especially these young might be vulnerable to the subsequent effects of testosterone: reduction of growth (Groothuis 1993, Fennell and Scanes 1992b), delay or modification of moult (in Ketterson *et al.* 1996, Ros *et al.* 1994, Düttmann *et al.* submitted), decrease of begging behaviour, and modification of the syrinx (Groothuis and Meeuwissen 1992). Therefore, if juvenile aggression is testosterone dependent, it is to be expected that also in these young testosterone profiles are finely tuned to the challenging situation.

In the black-headed gull (*Larus ridibundus*), chicks hatched from a clutch size of three never perform aggression towards siblings. These chicks are however remarkably aggressive early in life towards intruders on the territory. They grow up in dense colonies in which they are completely dependent on



A territorial chick chases off an intruding chick (A-C), and performs the Oblique display (D)

the parents for food during the first 8 weeks after hatching. In the course of the first 2 weeks the chicks begin to demand so much food that often both parents have to leave the territory simultaneously to forage. The chicks are then attacked by adult and juvenile birds which intrude in the territory to scavenge or to establish new territories. The response to these challenges consists of vigorous performance of aggressive behaviour (Groothuis 1989b).

Similar to the field situation, aggression is lacking between the members of small (pseudo) family groups composed of not more than five chicks of the same age and reared together from the first week onwards. However, high levels of aggression occur when such pseudo families are confronted with other families or with a model of an adult conspecific (Groothuis 1989a, 1989b). It is not known whether such temporary increases of aggressive behaviour are

based on temporary changes in plasma levels of testosterone. Clearly testosterone may play a role since aggressive behaviour of juvenile black-headed gulls is strongly promoted by testosterone treatment (Groothuis and Meeuwissen 1992). However, the direct temporal relation between aggressive behaviour, aggressive challenges, and levels of testosterone is still unknown. For example, in the experiments with young gulls aggressive behaviour was maintained after termination of the testosterone treatment. Although basal levels of testosterone were then low (Groothuis and Meeuwissen 1992), small temporary elevations in testosterone levels may have occurred. Therefore, we studied whether the challenge hypothesis as postulated for adult birds can be applied to the regulatory mechanism of aggressive behaviour in chicks, after a temporary exposure to elevated basal levels of testosterone.

Two experiments with black-headed gull chicks were carried out. In a first experiment we tried to mimic the experiment of Groothuis and Meeuwissen (1992) in a more natural set up. Basal levels of testosterone were tried to increase temporarily by exposing the birds to a period of territorial challenges. Short and long term consequences for basal levels of testosterone, and the aggressive response to a standard aggressive challenge were analysed. In a second experiment we analysed in a similar set up as the one of Groothuis and Meeuwissen the relation between aggressive challenges and short term changes in testosterone levels in the period when basal levels of the hormone were returned to low values.

Methods

Rearing conditions and animal handling

Black-headed gull chicks ($n = 100$) were regularly and randomly collected in the field 2–3 days after hatching and hand reared. From these collections small groups were formed of three to five peers to form pseudo-sibling families (families) mimicking the natural nest condition. Sex of the birds was not established because this would need invasive surgery that might influence the behaviour. It can not be a confounding factor in the analyses because gulls show hardly any sexual differentiation in social behaviour even in response to testosterone treatment (Terkel *et al.* 1976, Wingfield *et al.* 1982, Groothuis and Meeuwissen 1992). To facilitate individual recognition by the observer the chicks were individually marked on head or back with rhodamine or picric acid (ICN Biochemicals, Cleveland, Ohio, USA; chemicals were dissolved in acetone) and received a colour ring.

The families were held in indoor cages. Each family was visually isolated from other families. Dimensions of the cages were approximately 1×1 m

which is the average territory in the field. The floor was covered with straw. Each cage contained a 100 watt lamp positioned in the middle of the cage providing a temperature of approximately 37 °C. After 2 weeks the lamp was replaced by a 30 watt lamp. The lamp was removed when the chicks were 1 month of age. In addition light was provided on the basis of 16 h light, 8 h dark schedule. Food and water were available ad libitum. During the first 2 weeks chicks were fed with a moistened mixture of food pellets for trout (Trouvit, Trouw, Gent, Belgium) and food for fowl chicks (Sivo start, Bogena, Waalwijk, The Netherlands). This basic diet was daily supplemented with smelt (*Osmerus eperlanus*) and mashed hard-boiled chicken eggs. At 2 weeks of age, the diet was gradually shifted to dry trout pellets, with egg-mash added twice a week. A vitamin supplement (Calviet, UTD, Meppel, The Netherlands) was added weekly.

Testosterone was administered using silicon tubes (Medica BV, 's Hertogenbosch, The Netherlands; internal diameter: 1.0 mm, external diameter: 3.0 mm, length of column: 12 mm, closed on both ends with 1 mm silicon glue) packed with 8 mg crystalline testosterone (Diosynth, Oss, The Netherlands). These were implanted subcutaneously in the neck region under local anaesthesia with lidocaine (Xylocaine, Astra, Rijswijk, The Netherlands). The incision was closed with stitches.

For measuring levels of testosterone blood was drawn from the brachial wing vein with a heparin-rinsed needle and syringe within five minutes after capture of the bird. After centrifugation, plasma was stored at -30°C until analysis.

Experimental design experiment I

Eighteen families were used for the experiment. Each family was provided with a nest box for shelter and was given 2 weeks to become territorial. At 2.5 weeks after hatching most partitions between eight families were removed (grouped condition). This resulted in a combination of five families into one enclosure of 2 × 3 m and a combination of three families into another enclosure of 1 × 3 m. All eight families in the grouped condition consisted of three chicks. The remaining 10 families consisted of five chicks each and were left continuously isolated (isolated condition).

Aggressive behaviour of all birds was tested once every 2 or 3 days. Blood samples were collected from randomly selected chicks once in the first and once in the third week after removal of the partitions on a day of behavioural testing but independent of this test: first week: isolated condition $n = 6$, grouped condition $n = 8$; third week: isolated condition $n = 10$, grouped condition $n = 12$.

Experimental design experiment II

All families consisted of three chicks and were kept isolated to prevent aggressive interactions. The experimental group comprised three families of which the chicks received testosterone implants at 9 days after hatching (T group: $n = 8$, one chick died). Implants were removed after 10 days. The control group consisted of six chicks selected at random from six families and that were handled as the testosterone-treated chicks but were not implanted (C group). To assess the effectiveness of the implants plasma testosterone levels in blood samples of the T group collected at 7 days of testosterone treatment were compared with those of the T and C group collected in the first week after termination of treatment: T group $n = 8$, C group $n = 5$. These samples were drawn independent of aggressive challenges.

Behavioural tests were carried out at 2–3-day intervals from the end of the implantation phase at 2.5 weeks after hatching until the period the chicks were able to fledge at 7 weeks. Additional blood samples were collected from the T and C group at the end of the fifth week ($n = 7$, $n = 5$, respectively) and from the T group at the end of the sixth week ($n = 6$). The samples were drawn within 5–10 minutes after exposure of the chicks to an aggressive challenge.

Behavioural tests

To assess the responsiveness of the chicks with aggressive behaviour to an aggressive challenge, the context of an adult intruder into the territory was simulated. This was done by confronting chicks with a stuffed adult conspecific. This test was a slight modification of the standard stimulus test that was developed for young black-headed gulls by Groothuis (1989b). During this test the observer was standing in front of the open door of the cage and handled the model. Each test consisted of five short successive periods of 1 minute: (1) the model was held in front of the open door of the cage; (2) the model was held in the middle of the cage (the chicks then often moved to the nestbox at the back of the cage); (3) the model was held close to the birds and each bird was touched three times with the bill of the model; (4) as in (2); (5) as in (1). To avoid habituation, the model was continuously kept in motion by slowly moving the model in the horizontal plane and by gently shaking the model vertically. Behavioural observations were recorded on voice tape.

As a measurement of the responsiveness to an aggressive challenge, the frequency of the Oblique display was taken. This display is the most conspicuous and frequently performed aggressive display in black-headed gull chicks and consists of an erect posture accompanied by a loud call (see Groothuis 1989a, 1989b).

Radioimmunoassay

Testosterone concentrations were measured by radioimmunoassay as described by Verjans *et al.* (1973) with the modifications described by Dieleman *et al.* (1983). The main cross-reactivities were 49.7% for 5 α -dihydrotestosterone, 7.64% for 4-androstene-3 β ,17 β -diol, and 3.35% for androstenedione. The interassay coefficient of variation was 14%. The limit of quantitation was 0.05 ng testosterone / mL blood plasma.

Statistical treatment

The data of levels of testosterone and of frequencies of Oblique showed skewed distributions. In order to apply parametrical tests, Students' *t*-tests and Pearson regression, the data were logarithmically transformed in case of levels of testosterone, and Poisson transformed in case of Oblique frequencies (see Zar 1984). After transformation the data showed normal distributions. Two-sided *p* values were used with the border of significance set to *p* = 0.05.

Results

Experiment I

In the first week after removal of the partitions, levels of testosterone were significantly higher in birds in the grouped condition than in birds in the isolated condition (Fig. 1A) (two-sample *t*-test, *t* = 3.96, *df* = 12, *p* = 0.0019). The same was true for the frequency of Oblique display (Fig. 1B) (two-sample *t*-test, *t* = 2.40, *df* = 12, *p* = 0.034).

Two weeks later when the social structure between families in the grouped condition was stabilised, basal levels of testosterone of these birds had dropped and were not different from the level of the chicks in isolated families (Fig. 1A) (two-sample *t*-test, *t* = 1.33, *df* = 24, *p* = 0.20). However, the frequency of Oblique during a standard aggressive challenge in chicks of the grouped condition remained high, and was significantly higher than in the chicks in isolated families (Fig. 1B) (two-sample *t*-test, *t* = 2.92, *df* = 24, *p* = 0.0075).

Experiment II

Testosterone implants resulted in plasma levels of testosterone of 0.24 ± 0.03 (*sem*) ng/mL. This was significantly higher than basal levels of testosterone in the first week after removal of the implant: 0.07 ± 0.04 (*sem*) ng/mL (matched pairs *t*-test, *t* = 5.61, *df* = 7, *p* = 0.0008). It was also higher than basal levels of testosterone of the C group: 0.08 ± 0.01 (*sem*) ng/mL (two-sample *t*-test, *t* = 3.68, *df* = 7, *p* = 0.0079).

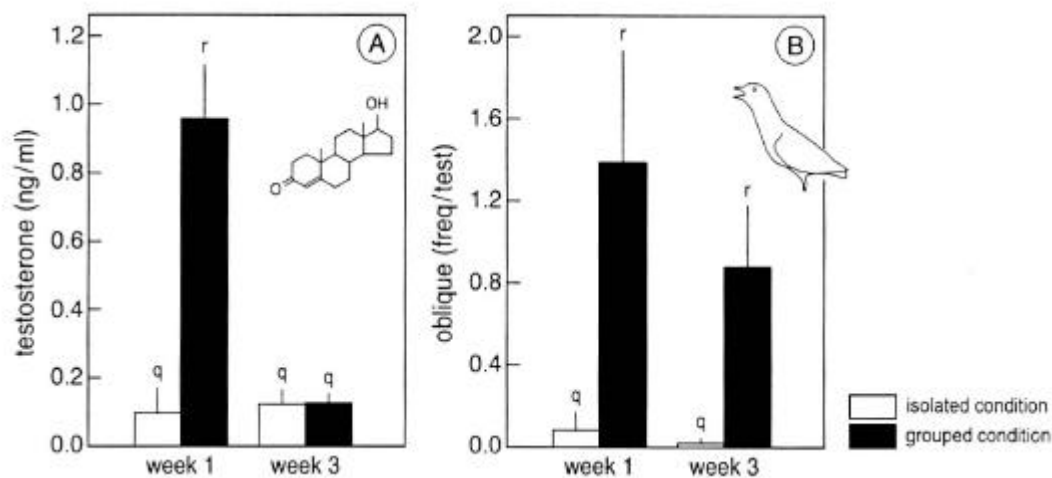


Fig. 1: Effects of social interaction between families of black-headed gull chicks on: (A) blood plasma levels of testosterone; (B) frequency of Oblique display. Plotted are *mean ± sem* values. At 2.5 weeks of age families of unrelated chicks were either confronted with other families in large enclosures (grouped condition) or were left isolated (isolated condition). Behaviour during the first week and the third week after re-housing the birds was observed during a standard aggressive challenge. Columns with different superscript (*q*, *r*) are significantly different.

During testosterone treatment birds of the T group performed more Oblique display than birds of the C group of the same age (Fig. 2) (two-sample *t*-test, day 18, $t = 2.20$, $df = 11$, $p = 0.05$). In the third week after termination of testosterone treatment Oblique was still significantly higher in the T group than in the C group (Fig. 2) (two-sample *t*-test, $t = 3.23$, $df = 11$, $p = 0.0080$) but in this period plasma concentrations of testosterone of both groups were at a similar low level (two-sample *t*-test, $t = 1.27$, $df = 11$, $p = 0.23$).

In the T group the level of testosterone after aggressive challenging was 0.46 ± 0.12 (*sem*) ng/mL. This was significantly higher than the basal level of testosterone in this group (matched pairs *t*-test, $t = 5.37$, $df = 6$, $p = 0.0017$). In the control group, the level of testosterone immediately after challenging was 0.75 ± 0.28 (*sem*) ng/mL, which was also significantly higher than their basal level of testosterone (matched pairs *t*-test, $t = 2.85$, $df = 4$, $p = 0.047$). The levels of testosterone after challenging did not differ significantly between the T and C group (two-sample *t*-test, $t = 0.38$, $df = 10$, $p = 0.71$).

At 47 days after hatching in the T group the response to the aggressive challenge waned to a level similar to that of the C group (Fig. 2: two-sample *t*-test, age 47, C group versus T group, $t = 1.42$, $df = 11$, $p = 0.18$; Fig. 3A: matched pairs *t*-test, birds of the T group, age 39 versus age 47, $t = 2.82$, $df = 5$, $p = 0.037$). At this age in four out of the six birds the level of testosterone was below the limit of quantitation. These birds also had the lowest response with Oblique display during the aggressive challenge test (Fig. 3).

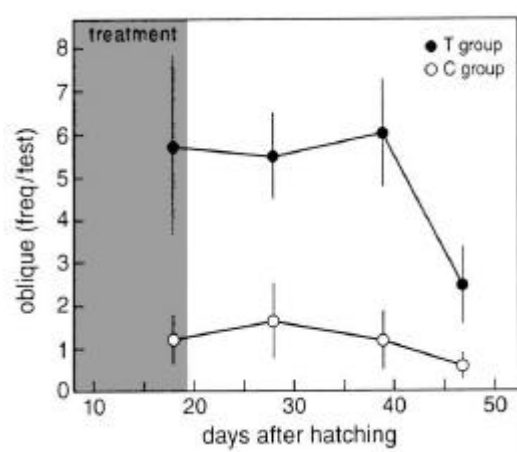


Fig. 2: Effect of testosterone treatment in black-headed gull chicks on the frequency of Oblique display during a standard aggressive challenge. Plotted are *mean* and *sem* values for testosterone-treated chicks (T group) and sham-treated control chicks (C group). Testosterone tubes were implanted at 9 days and removed at 19 days after hatching.

Discussion

The main aim of this paper is to test the challenge hypothesis for the regulation of aggressive behaviour early in life. Therefore we studied the relation between testosterone, aggressive challenges, and territorial behaviour in black-headed gull chicks. We demonstrate that testosterone plays an important role in facilitating this aggression by showing that: 1) untreated chicks that are active in territorial aggression had elevated basal blood plasma levels of testosterone; 2) the aggressive response to an aggressive challenge is highly increased in chicks treated with testosterone which confirms the result of an earlier study on young black-headed gulls (Groothuis and Meeuwissen 1992); 3) testosterone is quickly elevated when the chicks are aggressively challenged in a period when basal levels of the hormone are low. The relationship found between aggressive challenging, aggressive behaviour, and levels of testosterone is in agreement with the regulation of aggressive behaviour predicted on the basis of the challenge hypothesis.

Chicks which were exposed to experimentally increased levels of testosterone show persistently high levels of aggressive behaviour in response to an aggressive challenge even after termination of treatment when basal levels of testosterone were low. This was found by Groothuis and Meeuwissen (1992) for juvenile black-headed gulls. We now show that despite low basal levels of testosterone the chicks produced a short lasting elevation in testosterone plasma levels when they were aggressively challenged. This

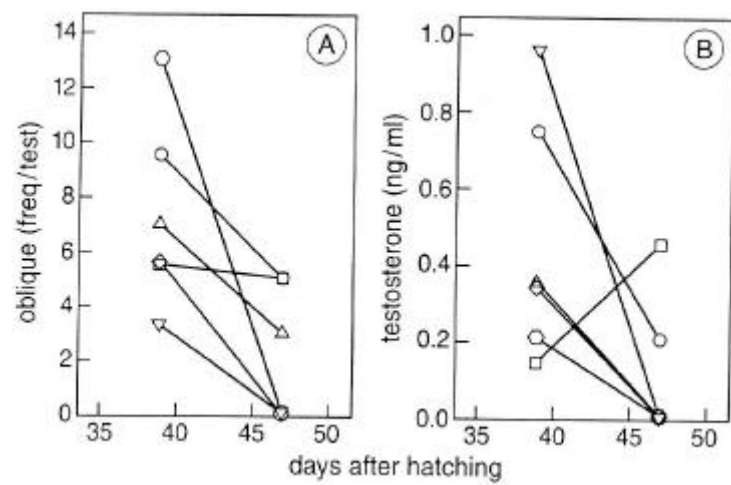


Fig. 3: Individual changes over age (after hatching) in: (A) frequency of Oblique display, and (B) blood plasma levels of testosterone, in black-headed gull chicks exposed to a 10 days treatment with testosterone. Behaviour was observed during a standard aggressive challenge. For measuring levels of testosterone during an aggressive challenge, blood was drawn within 5 minutes after termination of the standard aggressive challenge.

elevation was also found in untreated chicks. However, these birds showed relatively low levels of aggression when challenged in comparison to the chicks pretreated with testosterone. This suggests that exposure to testosterone increases the sensitivity of the aggressive response to the short lasting elevation in testosterone levels during aggressive challenges.

The results of both experiments showed many similarities. The levels of testosterone we induced by our testosterone treatment of chicks in isolated families (experiment II) were well within the range of testosterone levels found in untreated chicks in grouped families which can be taken as a model for the field situation (experiment I). In both experiments chicks show similar aggressive responses to a social challenge in the period basal levels of testosterone had returned to low values. We therefore assume that the increase in sensitivity of aggressive behaviour to short lasting elevations of testosterone found in chicks treated with testosterone plays an important role in the regulation of aggressive behaviour of black-headed gull chicks in the field.

Challenging with an aggressive stimulus only induced aggressive behaviour when the birds had experienced high levels of testosterone earlier. The effect of previous exposure to the hormone may be important for the relation between testosterone and behaviour as postulated in the challenge hypothesis. In monogamous species during the period the males are providing parental care basal levels of testosterone are low but a close association exists between aggressive behaviour and short lasting elevations in testosterone blood plasma levels during aggressive challenges (see Wingfield *et al.* 1990).

This may be an effect of the high levels of testosterone these males are exposed to in the beginning of the breeding season. In this way long-lasting effects of testosterone are important not only for sexual differentiation, but also for the development of individual differences within the same sex (see also van Oortmerssen *et al.* 1987, Moore 1991, Clark and Galef 1995).

It has been suggested that aggressive behaviour is only under the influence of testosterone when it is performed in a sexual context (see: Schwabl 1992, Wingfield and Monk 1992). So far studies testing the challenge hypothesis have mainly been carried out in adult birds during the reproductive period. We collected data from chicks which never showed any sign of sexual activity: they neither performed copulatory behaviour nor sexual displays. The results of our experiments clearly show that aggression outside the sexual context can be under the influence of testosterone. It has been proposed that the function of the mechanism postulated in the challenge hypothesis is that it enables a bird to perform aggressive behaviour but without constantly being exposed to high basal levels of testosterone. This is important for adult birds in the period when parental care is provided because it has been shown experimentally that this behaviour is incompatible with high levels of testosterone. This can obviously not be the adaptive value of the mechanism in the black-headed gull chicks. We suggest that the mechanism in black-headed gull chicks is adaptive because exposure to testosterone early in ontogeny suppresses growth. During the chick period selection for rapid growth will thus favour mechanisms that fine tune the level of testosterone exposure to the need to perform aggressive behaviour. Peaks in testosterone production early in ontogeny, probably resulting from interactions with conspecifics that intrude the nest territory, gradually increase the sensitivity to testosterone, thereby increasingly facilitating the behavioural responsiveness of the chicks.

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